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FOREWORD

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Julia Markett
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Introduction:

The Mouse Mammary Tumor Virus (MMTV) has been used as model system to study tissue specific gene expression. The MMTV retrovirus is transmitted both endogenously by proviruses inserted in the mouse genome and exogenously through virus particles secreted in the milk of an infected mouse. The highest expression of the virus is seen in the lactating mammary gland although a low level of expression occurs in salivary gland, testes and lymphoid tissue (1). This laboratory and others have previously mapped an enhancer element (designated Ban2) at the extreme 5' end of the 1.2 kb long terminal repeat (LTR) that has preferential activity in cell lines of mammary epithelial origin such as 34i (murine) or T47D (human) (2,3). In a previous study we showed that although the Ban2 enhancer contains several DNA elements for sequence specific transcription factors (AP-2, NF-1/CTF/F3, MAF/F12, and MP4) all of these proteins are expressed at similar levels in both mammary and non-mammary cell lines. Since all of the factors that bind to this tissue specific enhancer are ubiquitous we suggested that it is potentially the combination of all 4 factors and most importantly AP-2 and NF1/F3 that are required for the optimal expression of the LTR in mammary epithelial cells (3). In another study from this laboratory it was shown that the NF-1/F3 protein is related to but not identical to CTF1 and in fact may be a new family member. This protein termed F3 by our laboratory is being biochemically purified from HeLa cells (4). The results of the cloning and characterization of the gene encoding this protein will allow us to further investigate the protein-protein interactions that are required for activity of this enhancer.

In my original proposal I had suggested the use of transgenic mice to further study the tissue specific gene regulation of MMTV. However since there are many constructs that need to be tested, we decided that it would be more time and cost efficient to study the LTR in a more well defined tissue culture system. During the past year we initiated a collaboration with

the laboratory of Dr. Bissell at Lawrence Livermore Labs in Berkeley. Dr. Bissell's laboratory has published the description of a tissue culture system that closely models the lactating mammary gland. This in vitro system was developed to characterize milk protein gene regulation in lactating mammary gland (5). It is also useful to study how the mammary epithelial cells respond to their extracellular environment during the differentiation that occurs during lactation. It is now well established that the processes of development and differentiation depend on a cell's ability to sense self and its extracellular environment (6). A key extracellular component is the extracellular matrix (ECM). The ECM is an organized network composed of glycoproteins, proteoglycans and glycosaminoglycans which are important for cell morphology as well as transducing signals through cell surface integrins which ultimately leads to tissue specific gene expression (reviewed (7)).

In the adult animal, the gland develops and functionally differentiates in response to pregnancy. The mechanisms involved in this developmental process are complex and guided by various hormones (8), extracellular matrix (ECM) (9) and growth factors (10). Milk protein expression is initiated at mid-pregnancy and corresponds with the synthesis and deposition of ECM in alveolar development. Therefore, the expression of these milk proteins can be used as markers for the differentiated state of the gland. One in vitro model system for the study of ECM induced gene expression utilizes primary mammary epithelial cells (PMME) isolated from the mid-pregnant gland. PMME are able to respond to ECM by undergoing a three-dimensional reorganization to form an alveoli-like structure capable of synthesizing and vectorly secreting milk proteins into a central lumen. These alveolar-like structures are analogous to their in vivo counterparts in the lactating mammary gland (11). When an immortalized mouse mammary epithelial cell line CID-9, derived from the COMMA 1D cell strain, is cultured on ECM, it is able to form alveolar-like structures which are morphologically and functionally the

same as PMMEs (12). Dr. Bissell and colleagues have used stable transfections of CID-9 cells with the bovine b-casein promoter linked to chloramphenicol acetyl transferase (CAT) and determined that the transcriptional regulation of this gene is dependent on the presence of ECM and lactogenic hormones (12). Deletion analysis of the this promoter identified a 160bp transcriptional enhancer (BCE-1) capable of conferring ECM and hormonal regulation in either orientation when driving the inactive proximal b-casein promoter(-121 to +42)(13). We use the BCE-1 enhancer as a control for some experiments since it is known to require stable integration and ECM for its activity. They also showed preliminary results that MMTV is upregulated by ECM (14).

In this study we analyze the DNA elements in the MMTV LTR that are required for transcription in undifferentiated cells and mediate the response to differentiation on extracellular matrix in the presence and absence of hydrocortisone. We show that the 5' 150 bp of the LTR is required for optimal expression of the LTR and that the LTR is regulated by ECM. The elements that are required for the response to ECM map to the proximal 200 bp of the LTR which includes the glucocorticoid response elements (GRE's), NF1 and OTF1 binding sites. The MMTV LTR is upregulated by ECM only when the DNA is stably integrated into the CID-9 cells and we propose that it is a combination of chromatin organization and transcription factor binding that determines the activity of the MMTV LTR. The regulation of mammary epithelial cells by their extracellular environment most likely plays an important role in the high expression of MMTV in the lactating mammary gland. Since the upregulation of MMTV by ECM is shown to require a chromatin we tested whether the mechanism is altered by the presence of chemicals that modify histone structure by altering their acetylation state. It has been shown previously that sodium butyrate, an inhibitor of histone deacetylase, inhibits transcription from the MMTV LTR. In this study we test

the effect of sodium butyrate and a more specific inhibitor called Trichostatin A on the transcription of the LTR in both the presence and absence of ECM and hydrocortisone(15).

Body:

Experimental Methods

Plasmids for transient transfections:

MMTV/CAT, MMTV/b-cas/CAT, SV40/b-cas/CAT were described (12) LTR/Luc, B2/200-110/Luc are described (15)(4)respectively. The deletion constructs of LTRLUC (1070LUC, 870LUC, 380LUC) are described in Lefebvre et al.(15)

Cell passage and differentiation:

CID-9 cells (12) and their transfected derivatives were passaged in DMEM/F12, 5%FCS, 5ug/ml insulin (growth) and differentiated in DMEM/F12, 5ug/ml insulin(I) without or with 1ug/ml hydrocortisone(ih) and/or 3ug/ml prolactin(ip or ihp) as described in (13). Sodium Butyrate (Sigma Chemicals, St.Louis, MO) and Trichostatin A (Wako Pure Chemical Industry LTD,Richmond, VA) were prepared as a 100X stock in water and 1000X stock in ethanol respectively, the cells were treated 48 hours after plating and harvested 18 hours after treatment. In some of the experiments, Matrigel (Collaborative Biomedical Products, Bedford,MA) insulin(Gibco/BRL,Bethesda,MD) and prolactin (Sigma) were used. Stable transfections, cell harvest and CAT assays were performed an described in (13)

Transient transfections

CID-9 cells were plated in growth medium at a density of $.3 \times 10^6$ for a 60mm tissue culture plastic or 1.2×10^6 on 60mm dishes coated with .4mg polyhema(16) one day prior to transfection. The cells were changed to DMEM 5%FCS 5ug/ml insulin at least 3 hours before transfection. 10ug of test plasmid and 1ug RSV/bgal were co-transfected by the calcium

phosphate method (13) with the exception that precipitates were left on the cells for 18 hours. The cells were then washed 3x with DMEM/F12 and placed in differentiation media(ihp). The cells cultured on polyhema plates received differentiation media contained 2% Matrigel (17) Forty eight hours after differentiation, the cells were harvested with Dispase (12). Lysates were assayed for beta galactosidase activity(bgal) (Galacto-Light,Tropix), protein (Bio-Rad Protein Assay, Bio-Rad,Hercules,CA) and CAT (TLC) or Luciferase activity (15). CAT TLC plates were exposed to a PhosphorImager screen which was analyzed with a Molecular Dynamics PhosphorImager. The data is expressed as CAT density/bgal or Luciferase units/bgal units.

Results

Analysis of DNA elements required for MMTV expression in CID9 cells

The MMTV LTR and specific deletion constructs driving a luciferase reporter gene were transfected into CID9 cells and stable pools were generated by coselection with RSV-neo. The stable pools were tested for both the level of transcription and their response to being plated on ECM. Figure 1 shows compiled transcription data for 200LUC, Ban2/200LUC, 870LUC, 1070LUC, and LTRLUC stable pools. Figure 1 shows the basal (ip) and hydrocortisone induced (ihp) activity as measured by luciferase light units for each construct on plastic (1A) and on ECM (1B). The 200LUC construct which contains the minimal MMTV glucocorticoid responsive enhancer has a low level of activity that is stimulated 2-4 fold by hydrocortisone. Figure 1C is a bar graph of the data plotted to show the activity of each construct relative to 200LUC. Ban2/200LUC is induced 5 to 15 fold relative to 200LUC showing that Ban2 acts as an enhancer in these cells both in the basal and hydrocortisone induced state. The full length LTRLUC has a 12 to 40 fold higher activity than 200LUC. Interestingly, the two 5' deletions 1070 and 870 have a significantly lower activation level showing that something in

the first 100 base pairs of the LTR is required for optimal activity in CID9 cells. Ban2 acts as an enhancer as has been shown previously by our laboratory but since the 1070LUC construct contains the Ban2 fragment there must be additional elements 5' of Ban2 that are required for optimal expression.

Figure 1D shows the induction by ECM on the MMTV deletion constructs. Each construct shows 5 to 14 fold stimulation by plating the cells on ECM. This shows that the ECM activity is most likely contained within the 200 minimal enhancer and promoter. The ECM effect is not changed by the addition of hydrocortisone showing that the effect occurs in the uninduced basal state. In addition the hydrocortisone induction (1E) is unaltered by plating the cells on ECM showing that the MMTV sequences can be activated by hormone when plated in either plastic or ECM conditions. This data taken together with the data of Bissell and colleagues where the -206 to -66 MMTV enhancer linked to the minimal -121 b-casein promoter was induced 100 fold when cultured under Eihp conditions and 20 fold under Eih conditions, suggest that the ECM response of the MMTV LTR lies within the -200 minimal promoter (14). In order to determine what other factors may be mediating the upregulation of the promoter on ECM we analyzed the proteins that are known to bind to the LTR by mobility shift analysis. There are no visible changes in the mobility or quantity of OTF1, NF1/CTF, or AP2 in nuclear extracts isolated from CID9 cells in either growth or differentiation media and ECM (data not shown). This does not rule out the fact that there may be a modification of a factor or cofactor that is causing the upregulation of MMTV. In order to address this question we analyzed the MMTV LTRLUC and the 200LUC construct in transient transfection assays.

ECM does not induce transcriptional activation of transient templates

For transient transfection analyses we screened several enhancer-promoter constructs which when integrated into the

genome are known to respond either positively or negatively to the presence of ECM ((14) J. Michelotti and C. Myers unpublished data).

Figure 2 summarizes the transient and stable transcriptional activity of the MMTV, BCE-1 and SV40 enhancers linked to the bovine b-casein promoter, MMTV and BCE-1 enhancers linked to a minimal (-110bp) MMTV promoter as well as the full length MMTV LTR and a minimal (-200bp) MMTV promoter with or without the MMTV Ban2 enhancer(4). Briefly, ECM does not enhance BCE-1 induced transcription on a transient template even though endogenous b-casein is differentially expressed under these culture conditions as shown by immunoprecipitation of mouse milk proteins (data not shown). The basal b-casein promoter is capable of supporting transcription, as it is active when linked to the SV40 enhancer. The MMTV LTR which is induced up to 15 fold by the presence of ECM in stable pools, is not induced by ECM when assayed on the transient template. It does however retain some responsiveness to hydrocortisone. The requirement for stable integration of MMTV as well as the known role of chromatin reorganization in MMTV induction, led us to analyze the role of chromatin structure in ECM induced transcription.

The MMTV LTR is inhibited by deacetylase inhibitors in CID9 cells

Since the chromatin environment of the LTR is required for modulation by ECM we tested the effect of the deacetylase inhibitor sodium butyrate. It has been shown previously by this laboratory that the MMTV LTR is repressed by 5 mM sodium butyrate when the DNA is stably integrated (18).

Treatment of MMTV LTRLUC stably transfected CID-9 cells cultured in the presence of hydrocortisone and the absence or presence of ECM with sodium butyrate repressed transcriptional activity by 80-85% (Fig 3A & C). As Na butyrate treatment has been shown to induce many changes in the cell in addition to

inhibition of histone deacetylase (19, 20, 21, 22), we tested a more specific inhibitor of histone deacetylase Trichostatin A (23). A dose dependent repression of transcription was also observed in cells cultured in the absence or presence of ECM with Trichostatin A (Fig 3B & D). Trichostatin A repressed transcription 80% at a dose of 50 ug/ul in both growth conditions. To determine whether sodium butyrate and trichostatin A repression is due to a generalized decrease in cellular transcription, we analyzed the BCE-1 enhancer driving CAT in stably transfected CID-9 cells under the same culture conditions and treatments. Sodium butyrate as well as Trichostatin A treatment led to a dose dependent activation of BCE-1 transcription both in the absence and presence (Fig 3E & F) of ECM.

The LTRLUC CID9 cells were further tested on plastic to determine if this is an uninduced basal effect or one that requires hydrocortisone induction. Figure 4 shows the results of sodium butyrate and Trichostatin A treatment of CID9 LTRLUC cells in the absence (A) or presence (B) of hydrocortisone. Sodium butyrate inhibited LTRLUC 40% in the absence and 70% in the presence of hydrocortisone. Trichostatin A had the same effect of reducing activity 50 to 70% in either the basal or induced state. This data shows that the effect of deacetylase inhibitors occurs at the basal transcription level as well as in hormone induced cells. An earlier study from this laboratory used restriction enzyme access techniques to analyze sodium butyrate effects and did not fully address the effect in the basal state. In addition to confirming the previous work this data shows that the effect is most likely mediated through histone acetylation since a specific agent Trichostatin A has the identical effect as sodium butyrate.

In order to address which sequence elements in the LTR are required for the Trichostatin A repression, we tested several deletions of the LTR stably cloned into CID9 cells. Figure 4 shows the transcription data obtained from testing 870LUC in the

basal or induced state. Surprisingly, 870LUC is slightly activated in the absence of hydrocortisone (1.9 fold) and still repressed (53%) in the presence of hydrocortisone. Trichostatin A treatment has a similar pattern although the repression is slightly lower (42%) in the induced state. Preliminary experiments on further deletions show that the 380LUC construct has the identical profile as the 870LUC (Figure 4) further supporting the hypothesis that an element at the 5' end is mediating repression of the full length LTR by Trichostatin A. Further work is in progress to determine if the histone acetylation effect is mediated through specific sequences in the 5' end of the MMTV LTR. I will also continue studies to identify the sequences within the 5' 150 bp of the LTR that are required for the highest level of expression seen in CID-9 cells and the interaction of the proteins binding to the Ban2 enhancer that mediate tissue specific expression.

Discussion and Conclusion:

During the past year I have continued to study the question of how tissue specific expression of the MMTV LTR is established. Since all of the factors that bind to a well defined enhancer (Ban2) that has preferential activity in mammary epithelial cells are ubiquitous, I decided to look for additional mechanisms by which the LTR expression is enhanced or restricted. I have focused on two interesting mechanisms by which the LTR is regulated. The first is an upregulation of the MMTV LTR by the presence of factors that are present in the lactating mammary gland (eg. ECM). The second is a downregulation of the LTR by agents that cause accumulated histone acetylation which presumably alters chromatin structure.

There are several ways in which ECM could modulate MMTV gene expression. First the ECM could induce the levels or binding activity of NF1 or OTF1. However, in vitro EMSA indicates that these required factors are present and able to bind DNA independent of the ECM. This is in contrast to the ECM dependent

induction of albumin expression in hepatocytes where liver specific gene transcription depends on the presence of liver-enriched transcription factors which are upregulated in hepatocytes cultured on ECM(24).

Although in vitro binding does not address the functionality of proteins bound to the DNA, the lack of non-integrated MMTV constructs to respond to ECM suggests that cis-acting factor modifications are not sufficient to account for the upregulation of LTR transcription. Chromatin alterations caused by ECM which may lead to induction of gene expression may be achieved through any or all of the following: 1) histone modifications, 2) changes in DNA conformation, 3) factor accessibility, 4) DNA and/or factor nuclear localization (general chromatin reviews(25, 26)).

The fact that MMTV is repressed in the absence of ECM upon treatment with inhibitors of histone deacetylase suggests that histone modifications do play a role. However, the fact that the beta-casein BCE-1 enhancer (another ECM responsive gene) is activated by the same treatments suggests this is not a generalized phenomenon. One potential explanation is that MMTV requires an ordered histone/DNA interaction for proposed structural transitions which permit subsequent loading of necessary factors and activation of transcription (15). In a recent paper Beato and colleagues analyzed the effect of sodium butyrate and Trichostatin A on the MMTV LTR stably integrated into C127 (mammary epithelial derived) cells (27). They show that at low doses of acetylase inhibitors there is a slight activation of the LTR and it is only at higher doses that the LTR is repressed. They propose a model where a moderate level of histone acetylation enhances LTR transcription and that only when a heavy state of acetylation occurs is the LTR repressed. It is apparent in their paper that different cell types are more or less sensitive to the effects of sodium butyrate and Trichostatin A treatment. One cell type (T47D, human mammary epithelial) requires a higher dose of each drug to achieve a similar amount of histone acetylation. Since we used a different cell type

(CID9) in this study, it is difficult to directly compare data but the doses that we used in CID9 cells were similar to those that Beato and colleagues use. I have been unable to show activation of the LTR with either drug in CID9 cells which are a more relevant model for MMTV LTR gene expression. However, I have preliminary results in C127 derived cells that the LTR-LUC construct that we use is also upregulated at low doses of Trichostatin A confirming the work published by the Beato laboratory. I am in the progress of confirming whether there is a sequence in the LTR that is required for the modulation by Trichostatin A. Although factor availability, histone modifications or DNA accessibility alone can not account for ECM induced transcription, it is clear that complex interactions between several factors and their association with the chromatin template are involved.

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Appendices:

Figure Legends

Figure 1: Transfection data showing both the transcriptional properties and the ECM responsiveness of the various MMTV LTR constructs.

A shows the transcriptional activity of 200LUC, Ban2/200LUC, 870LUC 1070LUC and LTRLUC and is plotted as Luciferase units per ug of protein. This is an average of three different experiments on 2 to 3 separate stable pools of cells which were created by transfecting DNA into CID-9 cells and co-selecting with RSV-NEO for 2-3 weeks. **B** shows the identical constructs in stable pools when assayed after the cells have been plated on ECM as described in Materials and Methods.

C shows the activity of each construct as plotted relative to the activity observed with the minimal 200LUC construct. **D** is a bar graph showing the ECM induction for each construct which is a ratio of activity on ECM to activity on Plastic. **E** shows the data plotted as a ratio of hydrocortisone induced to uninduced for the same constructs.

Figure 2: ECM does not induce transcriptional activation of non-integrated templates in transient transfection analysis.

A diagram for each plasmid tested in transient transfection analysis is shown to the left. BCE-1, beta-ca and MMTV are labeled relative to the transcription start site of the endogenous genes.

The first six constructs contained the reported gene CAT and the last three contained luciferase(Luc). The adjacent table represents the activity of each construct when transiently or stably transfected into CID-9 cells and differentiated in (ihp) on plastic or ECM as in materials and methods. The transient activity is relative to co-transfected RSV beta galactosidase expression (a promoter which is not regulated by the presence of ECM). (-) represents no detectable activity and (+) represents increasing amounts of transcriptional activity. This data represents at least three independent transfections for each condition.

Figure 3: Treatment of CID-9 cells with deacetylase inhibitors has opposing effects on the BBC and MMTV LTR stable transfectants.

A, B, C and D are graphs of one of at least three experiments where MMTV LTR stable transfectants were plated on plastic or ECM in differentiation medium. The cells were cultured and treated as described above. The data is expressed as luciferase units per microgram of protein.

E and F are one of at least three independent experiments where BBC stably transfected CID-9 cells were plated on plastic and

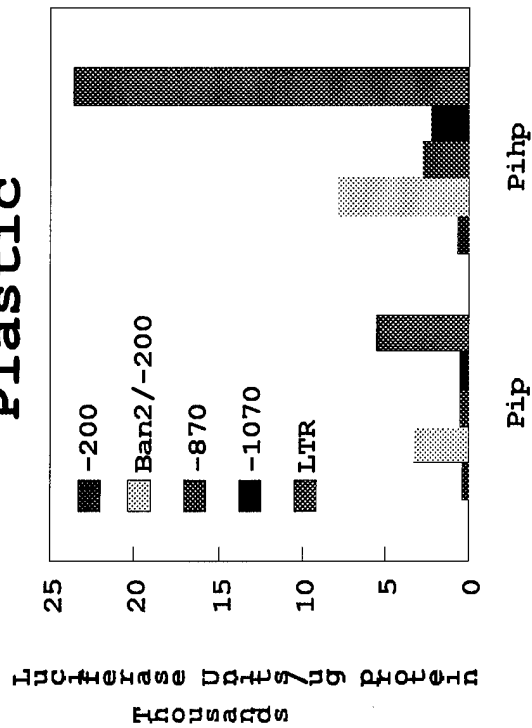
differentiated(ihp) for two days before harvest. Sodium butyrate or trichostatin A was added 18 hours before harvest. The autoradiography is of TLC separation of CAT activity for 10 ug of protein from cell lysates (**E & F**). The graph represents density per microgram protein per CAT reaction. (The above treatments will induce BBC activity of cells cultured on ECM about 2X)

Figure 4: Effect of acetylase inhibitors on two deletion constructs of LTRLUC.

Bar graphs of the activity of LTRLUC, 870LUC and 380LUC stable pools in CID-9 cells when treated with either Trichostatin A (**A**) or (sodium butyrate (**B**)). The cells were plated on plastic and grown in differentiation conditions in the absence (-) or presence (+) of hydrocortisone. The concentrations of Trichostatin A and sodium butyrate are 50 ug/ul and 5 mM respectively.

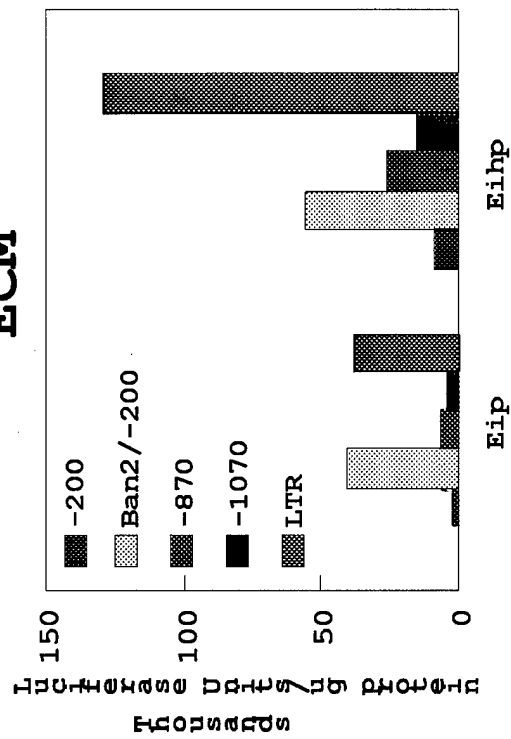
Figure 1

A **Plastic**

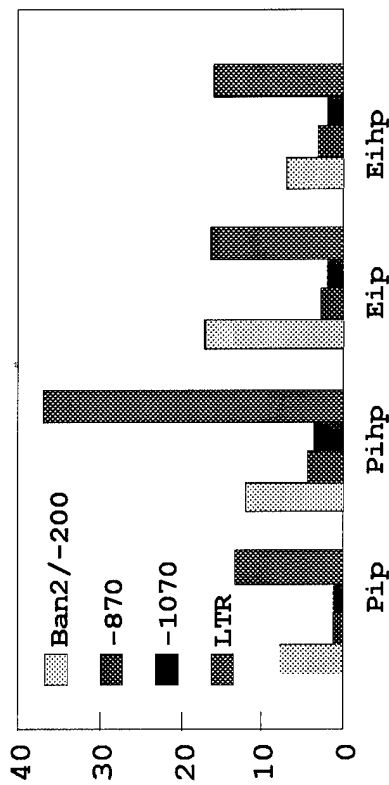


B

ECM

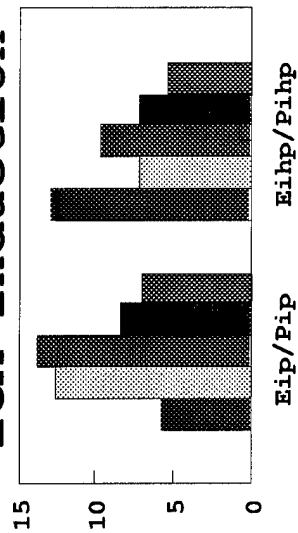


C **Fold over -200**



D

ECM induction



E

Hydrocortisone induction

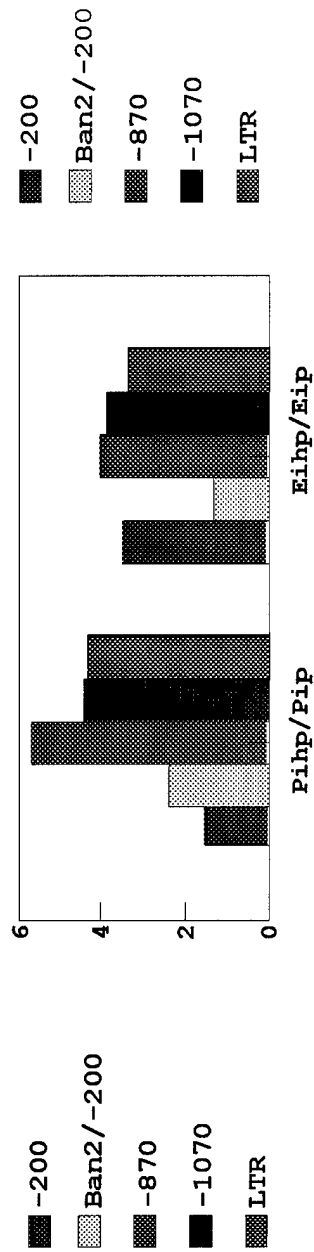


Figure 2

Transients		Stable	
Plastic	ECM	Plastic	ECM
-/+	-/+	-/+	+++++
+	++	+++	++
+	+/+	++	+++++
+	+/+	+	+/+
+	+	+	+++++
+	+	+	++++
++	++	++	++++
+	+	+	++
++	++	++	+++

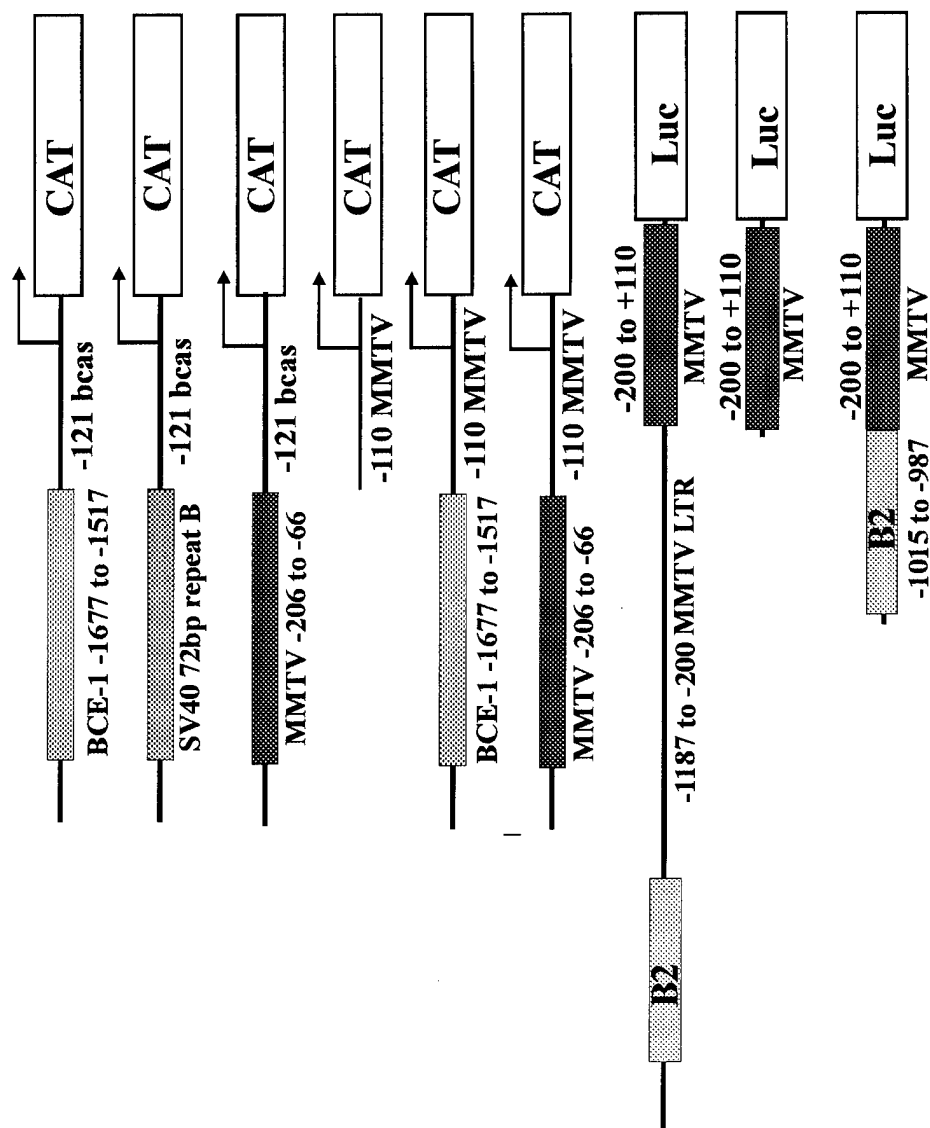
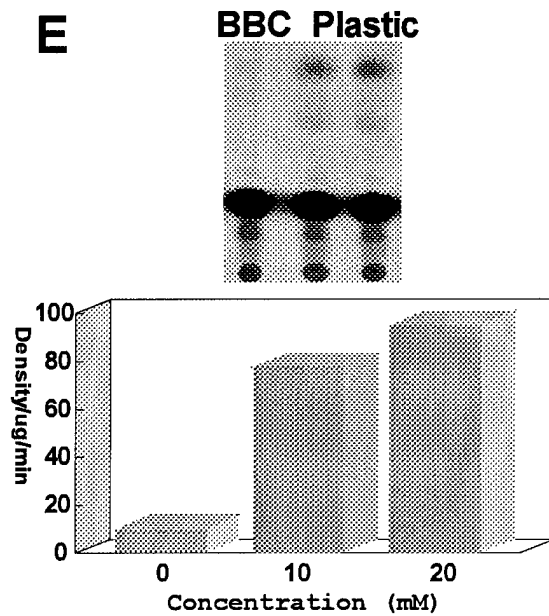
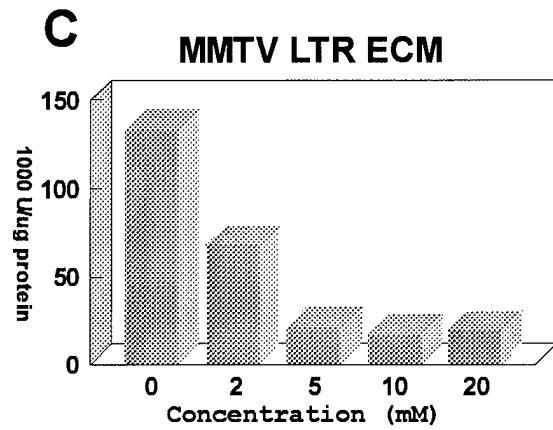
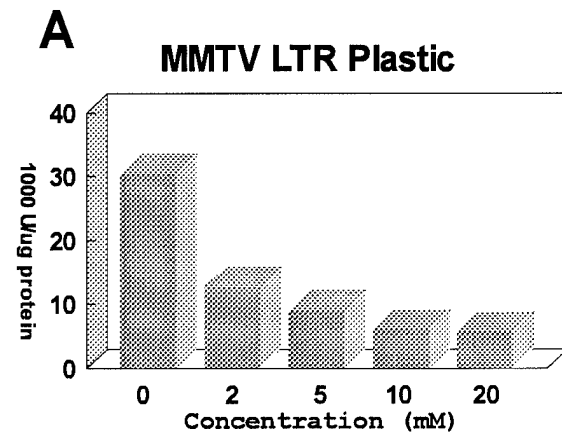


Figure 3

Sodium Butyrate



Trichostatin A

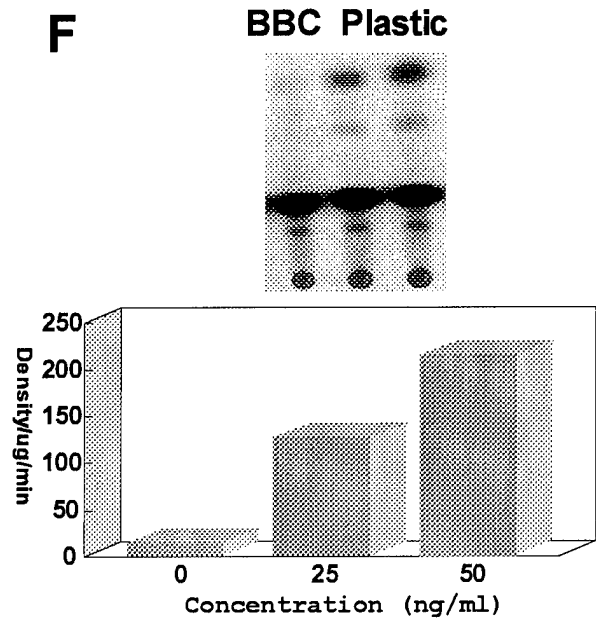
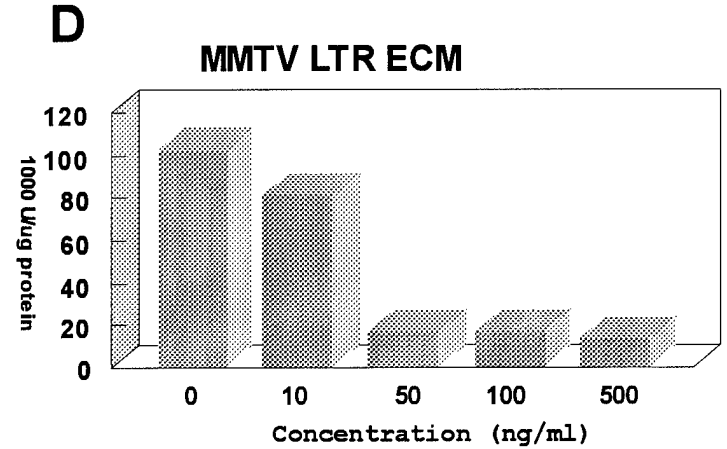
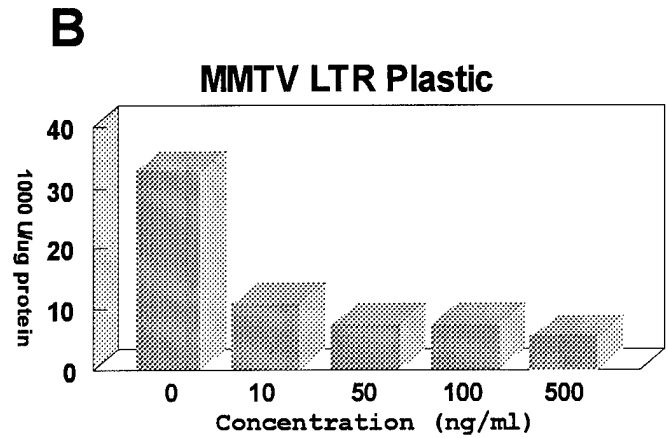
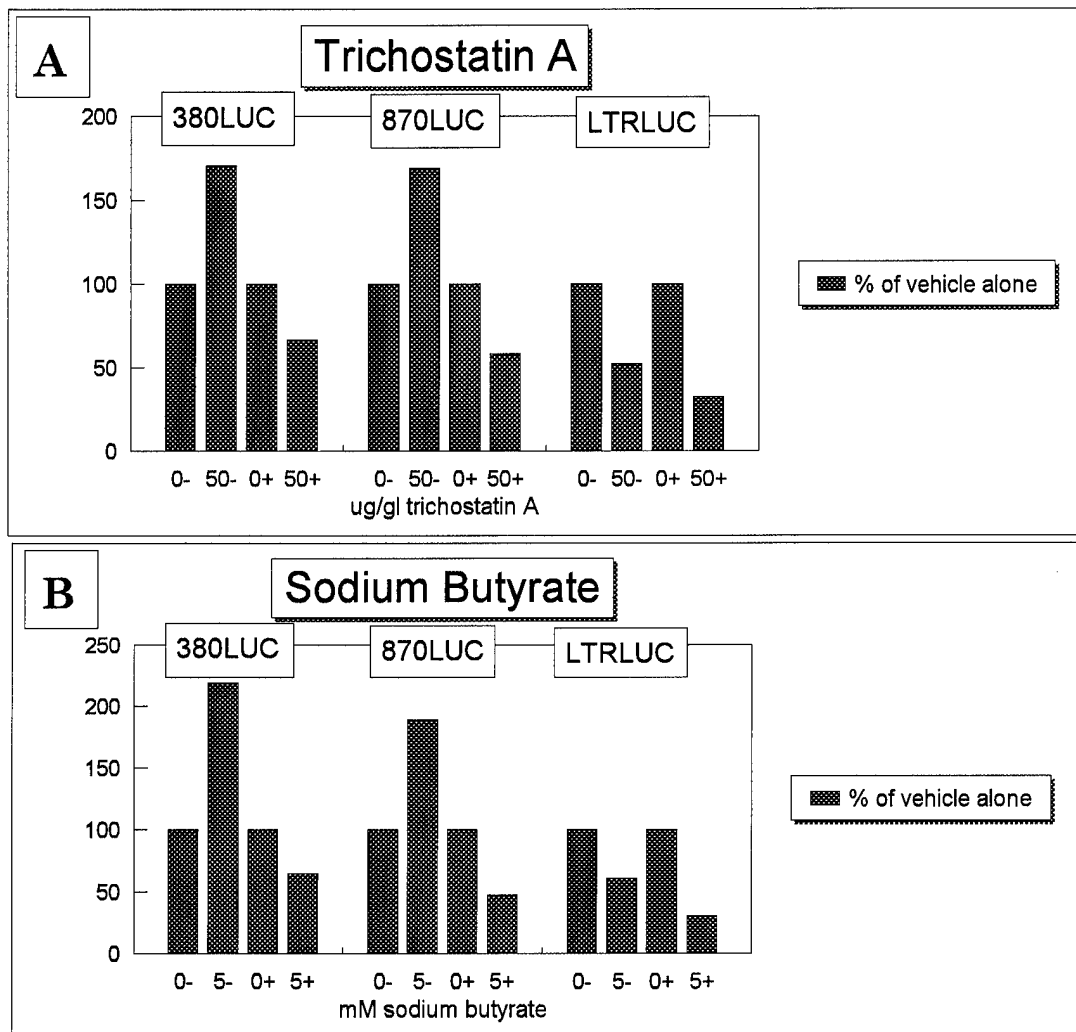


Figure 4



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Mellentin-Michelotti, J., S. John, W. D. Pennie, T. Williams, and G. L. Hager. 1994. The 5' enhancer of the mouse mammary tumor virus long terminal repeat contains a functional AP-2 element. *J. Biol. Chem.* **269**:31983-31990.

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Myers, C., C. Schmidhauser, J. Mellentin-Michelotti, G. Fragoso, G.L. Hager and M.J. Bissell. The BCE-1 enhancer from the bovine B-casein promoter requires integration in a complex chromatin for functional activation. *Mol Cell. Biol.* (in prep)

Mellentin-Michelotti, J., C. Myers, N. Malek, M.J. Bissell, and G.L. Hager. The MMTV promoter responds to ECM-induced differentiation of mammary cells when organized into replicated chromatin. *Development* (in prep)